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Three strains of *Wolbachia pipientis* and high rates of infection in Iranian sandfly species

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Abstract

Individual wild-caught sandflies from Iran were examined for infections of *Wolbachia pipientis* by targeting the major surface protein gene *wsp* of this intracellular α-proteobacterium. In total, 638 male and female sandflies were screened, of which 241 were found to be positive for one of three *wsp* haplotypes. Regardless of geographical origins and habitats, *Phlebotomus* (*Phlebotomus*) *papatasi* and other sandfly species were found to be infected with one common, widespread strain of A-group *W. pipientis* (Turk 54, GenBank accession EU780683; AY288297). In addition, a new A-group haplotype (Turk07, GenBank accession KC576916) was isolated from *Phlebotomus* (*Paraphlebotomus*) *mongolensis* and *Phlebotomus* (*Pa.*) *caucasicus*, and a new B-group haplotype (AZ2331, GenBank accession JX488735) was isolated from *Phlebotomus* (*Larroussius*) *perfiliewi*. Therefore, *Wolbachia* was found to occur in at least three of the incriminated vectors of zoonotic cutaneous leishmaniasis and zoonotic visceral leishmaniasis in different geographical regions of Iran. It may provide a new tool for the future control of leishmaniasis.

Keywords: *Wolbachia pipientis*, Iranian sandflies, *wsp* gene

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Introduction

Phlebotomine sandflies (Diptera: Psychodidae) of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World are the principal vectors of *Leishmania* to humans within their wide geographical range (Killick-Kendrick, 1990; Ready, 2013). The intracellular Rickettsia-like bacterium *Wolbachia pipientis* Hertig has been detected in sandflies (Benlarbi & Ready, 2003), and in this report we investigate its distribution in Iranian sandflies and the implications.

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Wolbachia are α-proteobacteria, purple, Gram-negative and maternally inherited bacteria (Saiful Islam, 2007; Brennan *et al.*, 2008). This bacterium is a reproductive parasite and a secondary symbiont thought to infect over 60% of insect species (Tanaka *et al.*, 2009; Perlman *et al.*, 2010). Only *Wolbachia* is known to induce four phenotypes of reproductive defects (cytoplasmic incompatibility (CI), male killing, feminization of genetic males and parthenogenesis induction), among which CI is the most common (Turelli & Hoffmann, 1999; Weeks *et al.*, 2002; Cordaux *et al.*, 2011). *Wolbachia* infections are found in many species of mites, crustaceans and insects, including sandflies (Ono *et al.*, 2001; Benlarbi & Ready, 2003; Hilgenboecker *et al.*, 2008; Wu & Hoy 2012).

More recently, the relatively fast evolving *Wolbachia* surface protein gene (*wsp*) has been used to improve phylogenetic resolution within the species clade of *W. pipientis*, which was divided into four groups (A–D) and 12 subgroups

Table 1. Number of *Paraphlebotomus* subgenus species screened for *Wolbachia* infections.

Genus	Subgenus	Species	Province	Golastan		(+ve) and Total	% (+ve) for each species (total)	% (+ve) subgenus infection (total)
			Regions Habitat	Gonbad kavous	Maraveh tapeh			
Phlebotomus	*Paraphlebotomus*	*P. sergenti*	ASH	10 (5)	12 (7)	(20) 44	45.45 (44)	9.85 (203)
			IH	2 (1)	2 (0)			
			RB	10 (4)	8 (3)			
		P. alexandri	ASH	10 (6)	0	(6) 10	60 (10)	2.95 (203)
			IH	0	0			
			RB	0	0			
		P. mongolensis (male)	ASH	27 (13)	13 (0)	(27) 85	31.76 (85)	13.30 (203)
			IH	5 (0)	10 (0)			
			RB	25 (14)	5 (0)			
		P. caucasicus (male)	ASH	5 (3)	0	(5) 7	71.43 (7)	2.46 (203)
			IH	0	0			
			RB	2 (2)	0			
		P. mongolensis/ P. caucasicus (female)	ASH	12 (3)[1]	20 (2)	(16) 57	28.07 (57)	7.89 (203)
			IH	3 (1)	7 (2)			
			RB	10 (7)[2]	5 (1)			
		Total		121 (59)	82 (15)	(74) 203	–	36.45

ASH, Animal shelter; IH, Inside houses; RB, Rodent burrow; +ve, *Wolbachia* positive.
[1] The presence of Turk 54 *Wolbachia* infected was determined by species, habitat and location from Duzalum Fadavi village of Gonbad kavous district.
[2] The presence of Turk 07 *Wolbachia* infected was determined by species, habitat and location from Okhi Tapeh village of Gonbad kavous district.

(Zhou *et al.*, 1998; Ono *et al.*, 2001). Groups A and B are concordant with those identified by 16S rDNA for the strains of *W. pipientis* from insects, mites and crustaceans, whereas groups C and D harbour the strains from filarial nematodes.

Traditionally, *Wolbachia* spp. detected in arthropods have been divided in two groups (*A* and *B*) based on sequences of the 16S rRNA, *ftsZ* and *wsp* genes (Werren *et al.*, 1995; Zhou *et al.*, 1998). It is worth noting that the sharing of *wsp* sequences between A and B strains indicates a strong genetic cohesiveness of *Wolbachia* strains, supporting designation of these bacteria within the same species, *W. pipientis* (Baldo *et al.*, 2006). Both groups contain *Wolbachia* spp. that has been detected in several genera of sandflies. Indeed, group A contains the *Wolbachia* species detected in *Sergentomyia minuta*, and *Wolbachia* species detected in *Phlebotomus*. Group B contains *Wolbachia* species detected in sandflies belonging to *Phlebotomus* and *Lutzomyia* genera (Werren *et al.*, 1995; Zhou *et al.*, 1998; Ono *et al.*, 2001). *Wolbachia* has been used recently to resolve the phylogenetic relationships among different *Wolbachia* strains. Based on *wsp* gene sequences from different *Wolbachia* isolates, it was proposed that the *Wolbachia A* and *B* clades be divided into 12 groups (Zhou *et al.*, 1998).

There have been very few reports of *wsp* gene being isolated and sequenced from *Wolbachia* of sandfly species using PCR to amplify a fragment of the *wsp* gene (Zhou *et al.*, 1998; Cui *et al.*, 1999; Ono *et al.*, 2001; Kassem *et al.*, 2003), in order to investigate the numbers of *W. pipientis* strains infecting wild populations of sandflies (Benlarbi & Ready, 2003; Parvizi *et al.*, 2003). The current report does this for *Phlebotomus papatasi* and subgenus *Paraphlebotomus* species from different zoonotic cutaneous leishmaniasis (ZCL) foci (Nadim & Seyedi-Rashti, 1971; Parvizi & Ready, 2008; Akhavan *et al.*, 2010; Motazedian *et al.*, 2010) and for subgenera *Adlerius* and *Larroussius* species from different

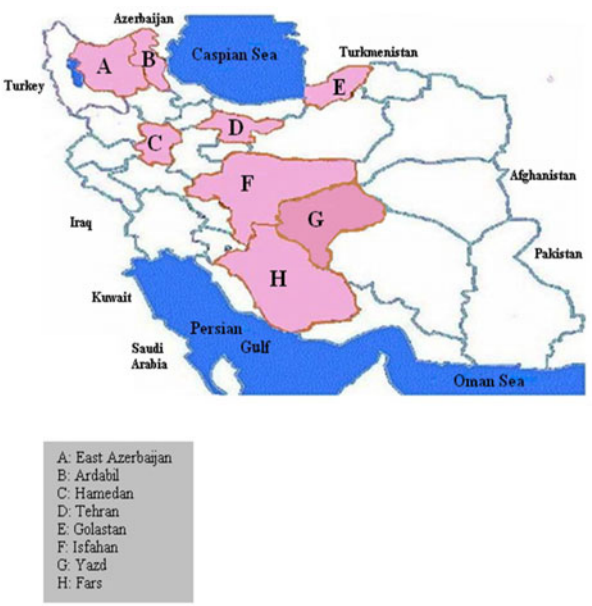

Fig. 1. Locations of Iranian provinces where sandfly species were sampled.

zoonotic visceral leishmaniasis (ZVL) foci (Nadim & Seyedi-Rashti, 1971; Nadim *et al.*,1992; Parvizi *et al.*, 2008), and it is an essential piece of information for assessing how *W. pipientis* might be used to drive transgenes through wild populations of this sandfly (Zhou *et al.*, 1998; Benlarbi & Ready, 2003).

Few infections of *W. pipientis* have already been identified only in *P. papatasi* in Iran. Therefore, more investigations on infection of this sandfly in a wider geographical range are

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Table 2. *Wolbachia* infections in *P. papatasi* from different habitats and various geographical locations in Iran.

Locations		Habitats																Total (+ve) % for each region	Total (+ve) % each region with all locations
Provinces	Regions	ASH		Inside houses								Outside houses				RB			
				Bath room		WC		Store room		Bed room		Yard		Stone crack					
		M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F		
Ardabil	Meshkin shahr	3 (1)	3 (0)	0	1 (0)	1 (1)	1 (1)	1 (1)	0	1 (0)	0	0	2 (1)	0	3 (1)	0	0	16 (6) 43	262 (6) 2.29
Isfahan	Isfahan	30 (25)	28 (25)	0	0	0	0	0	0	0	0	0	0	0	0	35 (25)	53 (32)	146 (107) 73.28	262 (114) 43.51
	Natanz	10 (1)	10 (4)	0	0	0	0	0	0	0	0	0	0	0	0	1 (1)	1 (1)	22 (7) 31.81	
Golastan	Gonbad kavous	7 (3)	3 (1)	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (0)	11 (4) 36.36	262 (9) 3.43
	Maraveh tapeh	0	3 (1)	0	0	0	0	0	0	0	2 (2)	0	0	0	2 (1)	0	2 (1)	9 (5) 55.55	
Fars	Shiraz	1 (0)	1 (1)	0	0	0	0	0	0	0	1 (0)	0	0	0	0	2 (0)	8 (5)	13 (6) 46.15	262 (6) 2.29
East Azerbaijan	Kaleybar	0	0	0	2 (0)	0	2 (1)	0	2 (1)	0	3 (0)	3 (0)	2 (1)	1 (0)	1 (0)	0	0	16 (3) 18.75	262 (3) 1.15
Hamedan	Hamedan	*	0	0	8 (7)	0	2 (2)	0	3 (2)	3 (3)	0	0	0	0	0	0	0	16 (14) 87.5	262 (14) 5.34
Tehran	Karaj	0	0	0	0	0	0	1 (1)	0	0	2 (2)	0	0	0	0	0	0	3 (3) 100	262 (3) 1.15
Yazd	Abarkouh	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10 (0)	10 (0) 0	262 (0) 0
Total		51 (30)	48 (32)	0	11 (7)	1 (1)	5 (4)	2 (2)	5 (3)	4 (3)	8 (4)	3 (0)	4 (2)	1 (0)	6 (2)	38 (26)	75 (39)	–	262 (155) 59.16

(+ve), *Wolbachia* positive; M, male; F, Female.

required. In addition, infection status needs to be established in other sandfly species, in which *W. pipientis* has never been recorded from Iran or elsewhere.

Material and methods

Sandflies were collected from villages in ten regions of eight provinces, using CDC traps and sticky papers (fig. 1). Sandflies were dissected. The head and genital terminalia of sandflies were kept for identifying species based on morphological characters. Thorax and abdomen were stored at −80°C until required for extracting DNA and PCR (Parvizi *et al.*, 2003).

About 550 base-pairs (bp) (minus primers) of the *wsp* gene were amplified by PCR using the primer pair *wsp* 81F (Forward) and *wsp* 691R (Reverse), with PCR amplification being carried out according to the protocol of Benlarbi & Ready (2003).

A 20 µl PCR reaction mixture consisted of 2 µl 1 × Promega buffer, 2 µl $MgCl_2$ 5 mM, 0.5 µl of each dNTP 0.25 mM, 1 µl of each primer 0.75 µl, 0.2 µl TaqDNA polymerase 0.05 unit/µl (Promega) and 2 µl of sandfly genomic DNA 1.5 mM. The PCR amplification was carried out with the following thermal profile using a GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems): 2 min. denaturation at 94°C; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 1 min. 30 s; and a final extension at 72°C for 10 min (Parvizi *et al.*, 2003).

After amplification, the samples were fractionated by horizontal submerged gel electrophoresis, using 1.5% agarose gels and DNA size markers (Promega PCR markers G316A or Bioline Hyper ladder IV). DNA fragments were visualized by ethidium bromide staining, then excised and purified using a Geneclean II Kit (BIO 101 Inc) before cycle sequencing each strand. The sequences obtained were edited and aligned with database sequences using SequencherTM 4.1.4 software to identify unique sequences (=haplotypes), which were analysed phylogenetically using MEGA 4 (Tamura *et al.*, 2007).

Results

Three haplotypes of *wsp* gene from Iranian sandflies were found. Haplotype Turk 54 was identified in two GenBank sequences from strains of *W. pipientis* isolated from *P. papatasi* originating from Iran and Egypt (GenBank accession EU780683; AY288297) (table 2), and it predominated in Iranian sandflies infected with *W. pipientis* (144/158 infections). Two new haplotypes, Turk 07 (10/158 infections) and AZ2331 (4/158 infections) of *W. pipientis* were identified in Iranian sandflies (fig. 2). The haplotype Turk07 (GenBank accession number KC576916) was isolated from two males of *Phlebotomus mongolensis*, two males of *Phlebotomus caucasicus* and six females of *P. mongolensis*/*P. caucasicus* originating from Turkemen Sahara, Iran. The haplotype AZ2331 (GenBank accession number JX488735) was isolated from *Phlebotomus perfiliewi* originating from East Azerbaijan province, and it was distinguishable from a sequence previously submitted in GenBank (AF237884 strain *wPrn* of *W. pipientis*) isolated from *Phlebotomus perniciosus* originating from Italy only by having an ambiguous base (G/A) at nucleotide position 18.

Populations of sandflies from different regions and habitats originating from areas endemic and non-endemic for ZCL and ZVL in Iran were screened by PCR for *W. pipientis* infections using the species-specific but non-strain specific

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Table 3. *Wolbachia* infections in two subgenera species using *wsp* gene in three endemic visceral leishmaniasis locations in North West of Iran.

Subgenus	Provinces	Ardabil	East Azerbaijan		Total (+ve) % for each species	Total (+ve) % for each subgenus
	Species Regions	Meshkin shahr Total (+ve)	Sarab total (+ve)	Kaleybar total (+ve)		
Larrossius	*P. kandelakii*	26 (1)	14	10	50 (1) 2	103 (10) 9.70
	Phlebotomus tobbi	0	5	0	5 (0) 0	
	P. perfiliewi	3	14 (2)	24 (7)[1]	41 (9) 21.95	
	P. major	5	2	0	7 (0) 0	
Adlerius	*P. simici*	0	7	0	7 (0) 0	80 (2) 2.5
	Phlebotomus brevis	0	1	0	1 (0) 0	
	Phlebotomus halepensis	7	21	0	28 (0) 0	
	Phlebotomus longidoctus	6	0	1	7 (0) 0	
	Phlebotomus balcanicus	0	1	0	1 (0) 0	
	Adlerius Female	10	15	11 (2)	36 (2) 5.55	
	Total	57 (1)	80 (2)	46 (9)	_	183 (12) 6.56

[1] New *Wolbachia* infected haplotype AZ2331 GenBank ID. JX488735.

Fig. 2. Unrooted neighbour-joining tree showing the relationships of the haplotypes of the *Wolbachia* surface protein gene (*wsp*) fragment found in Iranian sandflies and GenBank.

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Table 4. Numbers and percentages of *Wolbachia* infections (*wsp* gene) in sandfly species screened by PCR from different regions in Iran.

Genera			*Phlebotomus*																
Subgenus			*Larrossius*			*Adlerius*							*Paraphlebotomus*						
Species			*P. kandelakii*	*P. Tobbii*	*P. perfiliewi*	*P. major*	*P. simici*	*P. brevis*	*P. halepensis*	*P. longidactus*	*P. balcanicus*	*Adlerius* female species	*Phlebotomus P. papatasi*	*P. sergenti*	*P. alexandri*	*P. mongolensis (male)*	*P. caucasicus (male)*	*P. mongolensis / P. caucasicus (female)*	Total (infection)	Each location infection (%)
Provinces/regions																				
East	Kaleybar		10	0	24 (7)[1]	0	0	0	0	1	0	11 (2)	16 (3)	0	0	0	0	0	**62 (12)**	9.85
Azerbaijan	Sarab		14	5	14 (2)	2	7	1	21	0	1	15	0	0	0	0	0	0	**80 (2)**	
Ardabil	Meshkin shahr		26 (1)	0	3	5	0	0	7	6	0	10	16 (7)	0	0	0	0	0	**73 (8)**	10.95
Isfahan	Isfahan		0	0	0	0	0	0	0	0	0	0	146 (107)	0	0	0	0	0	**146 (107)**	67.85
	Natanz		0	0	0	0	0	0	0	0	0	0	22 (7)	0	0	0	0	0	**22 (7)**	
Golastan	Gonbad kavous		0	0	0	0	0	0	0	0	0	0	11 (4)	23 (10)	10 (6)	67 (27)	6 (5)	32 (11)[2]	**149 (63)**	36.77
	Maraveh tapeh		0	0	0	0	0	0	0	0	0	0	9 (4)	21 (10)	0	18	1	25 (5)	**74 (19)**	
Fars	Shiraz		0	0	0	0	0	0	0	0	0	0	13 (6)	0	0	0	0	0	**13 (6)**	46.15
Tehran	Karaj		0	0	0	0	0	0	0	0	0	0	3 (3)	0	0	0	0	0	**3 (3)**	100
Hamedan	Hamedan		0	0	0	0	0	0	0	0	0	0	16 (14)	0	0	0	0	0	**16 (14)**	87.50
Yazd	Abarkouh		0	0	0	0	0	0	0	0	0	0	10 (0)	0	0	0	0	0	**10 (0)**	0
Total			50 (1)	5	41 (9)	7	7	1	28	7	1	**36 (2)**	**262 (155)**	**44 (20)**	**10 (6)**	**85 (27)**	**7 (5)**	**57 (16)**	**648 (241)**	**37.19**

() = *Wolbachia* positive.
[1] The presence of AZ2331 (accession number, JX488735) *Wolbachia* infected was caught from ASH determined by species, region and province.
[2] The presence of Turk 54 (accession number, EU780683; AY288297) and Turk 07 (accession number, KC576916) were caught from ASH and RB, respectively determined by species, region and province.

primers for the *wsp* genes. In all collections, 203 males and females of the subgenus *Phlebotomus (Paraphlebotomus)* were identified from different habitats in 13 villages from two districts of Turkemen Sahara, Golastan province, Iran (table 1): 74 out of the 203 specimens were found with *Wolbachia* infections; and all four species were infected (*Phlebotomus sergenti* 20/44, *Phlebotomus alexandri* 6/10, *P. mongolensis* 27/85, *P. caucasicus* 5/7 and *P. mongolensis*/*P. caucasicus* 16/57) (table 1). The females of *P. mongolensis* and *P. caucasicus*, as well as the females of all *Adlerius* species in Iran, could not be separated morphologically based on the structure of the spermathecae or the weakly developed pharyngeal armature (Theodor & Mesghali, 1964; Parvizi *et al*., 2010*a*, *b*).

Many males and females of *P. papatasi* were identified and chosen from different habitats in 62 villages from ten districts and eight provinces of Iran (table 2). Of all *P .papatasi* screened for the *wsp* gene, 155/262 were positive, with infections found in seven provinces and most originating from Isfahan and Hamedan provinces (table 2).

Of the four *Larrossius* and five *Adlerius* species identified, 183 males and females were screened for *wsp* from 12 different villages in three districts of Ardabil and East Azerbaijan provinces, Iran: *P.* (*La.*) *perfiliewi* (9/41), *Phlebotomus* (*La.*) *kandelakii* (1/50) and *Adlerius* species (2/80, both females) were positive (table 3).

In total, 638 male and female Iranian sandflies were screened for *wsp* gene, and 241 of them were positive (table 4). However, only 158 out of 241 (65.56%) of these PCR products contained enough DNA for successful direct sequencing. Three haplotypes of the *wsp* gene were obtained from Iranian sandflies. The three sequences were aligned with some *wsp* sequences of *Wolbachia* from sandflies and other insects (Matsumoto *et al*., 2008; Azpurua *et al*., 2010; Henri & Mouton, 2012), and phylogenetic relationships were generated via neighbour-joining analysis using MEGA software (fig. 2). The common haplotype (Turk 54) and the new haplotype Turk 07 are in the A-group and a new haplotype AZ2331 isolated from *P. perfiliewi* is in the B-group of strains of *W. pipientis*.

Discussion

Previously, we had identified a single strain of *W. pipientis* in wild-caught *P. papatasi* from Iran by targeting the *wsp* gene (Parvizi *et al*., 2003). But some unanswered questions included the possible presence of other strains that might have been missed if the PCR primers exhibited haplotype specificity (Mitsuhashi *et al*., 2004). Also, the screening of more sandfly species and specimens from different habitats and locations might have revealed a greater diversity of *wsp* gene haplotypes. This has now been carried out for the first time in Iran, revealing the presence of the *wsp* gene in four *Paraphlebotomus* species (*P. sergenti*, *P. alexandri*, *P. mongolensis* and *P. caucasicus*), two *Larroussius* species (*P. perfiliewi* and *P. kandelakii*) and females of *Adlerius* species. The infection rates were significantly higher in Isfahan compared with other locations sampled (χ^2 test: $P<0.05$) (tables 1 and 2). The overall ratio of infection rates within animal shelters (ASH) and rodent burrows (RB) in Golastan Province were significantly higher than elsewhere (χ^2 test: $P<0.05$) and most infections were found in ASH (tables 1 and 2).

Three haplotypes of the *wsp* gene were obtained (tables 1 and 4). Two were new (GenBank accession numbers KC576916 and JX488735), indicating new strains of *Wolbachia*. Regardless of geographical origins and habitat,

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most wild sandfly species mentioned in this report have been found infected with one common, widespread strain of *W. pipientis* (Turk 54, GenBank ID. EU780683; AY288297). The 564-bp haplotype (minus primers) had good and readable sequences. It was distinguished as an A-group strain of *W. pipientis* (wPap), and it was previously isolated from *P. papatasi* originating from Israel/West Bank (AF237883) (Ono *et al.*, 2001) and India (GenBank accession number AF237882 (Ono *et al.*, 2001), as well as from Spain and Iran (Benlarbi & Ready, 2003; Parvizi *et al.*, 2003). In addition, a new haplotype Turk07, isolated from *P. mongolensis* and *P. caucasicus*, is also in the A-group, in contrast to the new haplotype AZ2331 isolated from *P. perfiliewi* which is in the B-group of strains of *W. pipientis*. The latter differs by only one nucleotide from strain *wPrn* (GenBank ID AF237884) isolated from *P. perniciosus* in Italy (fig. 2) (Rasgon & Scott, 2003; Matsumoto *et al.*, 2008; Azpurua *et al.*, 2010; Henri & Mouton, 2012; Parvizi *et al.* 2013*a*,*b*). Therefore, we can conclude that more than one A-group strain of *W. pipientis* occurs in sandfly species in Iran.

The geographical and species distributions of the three *Wolbachia* strains in Iran are likely to depend on horizontal transmission (Benlarbi & Ready, 2003). This raises the possibility of using *W. pipientis* to drive transgenes through wild sandfly populations, with the objective of intervening in the transmission of *Leishmania*. This can be done by genetic engineering techniques followed by the mass release of transgenic insects. Such an approach could be very useful for the biological control of a variety of parasites and viruses (Werren, 1998; Dobson *et al.*, 2002; Mitsuhashi *et al.*, 2002; Rasgon *et al.*, 2003; Parvizi *et al.*, 2009).

The *wsp* gene is a very useful tool for typing different *Wolbachia* strains (Zhou *et al.*, 1998; Weeks *et al.*, 2002; Werren *et al.*, 2008). It is notable that *wsp* gene sequences have almost ten times greater divergence than the 16S rDNA sequences of *Wolbachia* from different host taxa (O'Neill *et al.*, 1992; Rousset *et al.*, 1992), which comparatively makes it the fastest evolving of five ubiquitous *Wolbachia* genes (gatB, coxA, hcpA, fbpA and ftsZ) among insect species (Zhou *et al.*, 1998; Baldo *et al.*, 2006). *wsp* typing is analogous to antigen protein typing used for pathogenic bacteria (Perez-Losada *et al.*, 2005). Its widespread use as a reliable marker for strain typing is also supported by its presumed role in host–symbiont interactions (Zhou *et al.*, 1998; Van Meer *et al.*, 1999; Pintureau *et al.*, 2000; Baldo *et al.*, 2002; Shoemaker *et al.*, 2002; Nirgianaki *et al.*, 2003; Kyei-Poku *et al.*, 2005). The use of more polymorphic *Wolbachia* sequences (Baldo *et al.*, 2006; Siozios *et al.*, 2013) could improve strain identification in sandflies. So far, strain identification has depended on *wsp* sequencing, with relatively few isolations from *Lutzomyia shannoni* Dyar (Colombia), *Lutzomyia trapidoi* (Panama), *Lutzomyia whitmani* Coutinho & Antunes (Brazil), *Lutzomyia vespertilionis* (Panama), *P. papatasi* Scopoli (India, Iran and Israel), *P. perniciosus* Newstead (France and Italy) and *S. minuta* (France) (Zhou *et al.*, 1998, Ono *et al.*, 2001, Matsumoto *et al.*, 2008; Azpurua *et al.*, 2010).

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